A vertebrate crossveinless 2 homologue modulates BMP activity and neural crest cell migration

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Summary

Previous work has revealed that proteins that bind to bone morphogenetic proteins (BMPs) and inhibit their signalling have a crucial role in the spatial and temporal regulation of cell differentiation and cell migration by BMPs. We have identified a chick homologue of *crossveinless 2*, a *Drosophila* gene that was identified in genetic studies as a promoter of BMP-like signalling. Chick Cv-2 has a conserved structure of five cysteine-rich repeats similar to those found in several BMP antagonists, and a C-terminal Von Willebrand type D domain. Cv-2 is expressed in the chick embryo in a number of tissues at sites at which elevated BMP signalling is required. One such site of expression is premigratory neural crest, in which at trunk levels threshold levels of BMP activity are required to initiate cell migration. We show that, when overexpressed, Cv-2 can weakly antagonise BMP4 activity in *Xenopus* embryos, but that in other in vitro assays Cv-2 can increase the activity of co-expressed BMP4. Furthermore, we find that increased expression of Cv-2 causes premature onset of trunk neural crest cell migration in the chick embryo, indicative of Cv-2 acting to promote BMP activity at an endogenous site of expression. We therefore propose that BMP signalling is modulated both by antagonists and by Cv-2 that acts to elevate BMP activity.

Key words: BMP activity, Neural crest, Cell migration, Chick, *Xenopus*

Introduction

During development, the establishment of organised patterns of tissues and cell types requires precise temporal and spatial control of cell proliferation, differentiation and movement. Extracellular protein ligands play a central role in cell-cell signalling required for patterning by binding specific cell-surface receptors and activating intracellular signal transduction pathways. Such signals can act in an autocrine manner to regulate the phenotype of the cells that generate the signal or can act in a paracrine manner on cells adjacent to or many cell diameters from the signalling source. An increasing number of mechanisms have been shown to modulate precisely the activity of intercellular signals, reflecting the importance of levels of particular signals. For example, a single morphogen can elicit differentiation of several distinct cell types at different threshold concentrations. The amount or spatial distribution of signalling activity can be regulated by: movement of a signal away from a localised source; inhibition of a widespread signal by localised extracellular antagonists; or induction of specific extracellular antagonists by the signal itself, creating a negative feedback loop (Moghal and Sternberg, 1999; Perrimon and McMahon, 1999).

The control of bone morphogenetic protein (BMP) ligand action is an excellent example of the modulation of cell-cell signalling. BMPs bind to and activate serine/threonine kinase receptors, leading to phosphorylation of SMAD transcription factors (Moustakas et al., 2001). They are important regulators of cell differentiation in many tissues and play a key role during early embryogenesis as revealed by studies in *Xenopus* showing that localised inhibition of BMP4 activity is required for the formation of neural ectoderm and dorsal mesoderm (De Robertis et al., 2000). This inhibition is mediated by multiple polypeptide antagonists secreted by the Spemann organiser, including follistatin, noggin and chordin, that bind to BMP4 and prevent its interaction with BMP receptor (Balemans and Van Hul, 2002). Further aspects of the control of BMP activity have been revealed by detailed studies of *Xenopus chordin* and its *Drosophila* homologue, *short gastrulation*. Formation of a complex between chordin and BMP4 blocks interaction of ligand with BMP receptor; however, following cleavage of chordin by the protease Xolloid, BMP4 is released and can bind to receptor (Piccolo et al., 1997). Another protein, *twisted gastrulation*, has a dual role in enhancing antagonism by stabilising chordin-BMP4 binding, and in promoting BMP4 activity by destabilising the binding of chordin cleavage fragments to BMP4 (Chang et al., 2001; Larrain et al., 2001; Oelgeschlager et al., 2000; Ross et al., 2001; Scott et al., 2001).

Following neural induction, BMP4 has a number of important functions in the formation and differentiation of the neural crest. This migratory population of cells contributes to many tissues in the vertebrate embryo, including melanocytes,

peripheral nervous system and the head skeleton. The neural crest is induced at the interface between neural epithelium and surface ectoderm. They subsequently leave the neuroepithelium by undergoing an epithelial-mesenchymal transition and migrating away from the neural tube, initially in the anterior and then in progressively more posterior parts of the embryo (Kalcheim, 2000; Knecht and Bronner-Fraser, 2002). BMP4 has been implicated in multiple steps of neural crest development: as a component of the signalling that induces neural crest (Knecht and Bronner-Fraser, 2002), in control of the onset of neural crest migration (Sela-Donenfeld and Kalcheim, 1999), in apoptosis of cranial neural crest (Graham et al., 1994) and later in the differentiation of neural crest to sympathetic neurons (Anderson et al., 1997). Modulation of the level of BMP4 activity appears to be important for the onset of trunk neural crest migration in the chick embryo. Following closure of the chick neural tube, BMP4 expression occurs in premigratory neural crest at similar levels along the anteroposterior (AP) axis, whereas the BMP4 antagonist noggin is expressed in a graded fashion – high posterior and low anterior. The onset of neural crest cell migration requires a threshold level of BMP4 activity, such that the progressive decrease of noggin expression underlies the anterior to posterior wave of migration (Sela-Donenfeld and Kalcheim, 1999).

Although many modulators of BMP activity have been identified, the function of only a subset has been established in the early vertebrate embryo. Furthermore, these factors appear to function by blocking BMP activity by interfering with binding to the receptor. Here, we identify a chick homologue of the *Drosophila* gene *crossveinless 2* (*cv-2*) and analyse whether it modulates BMP activity. *cv-2* encodes a protein with five cysteine-rich (CR) repeats found in BMP-binding proteins and is expressed in a number of sites of BMP4 action, including premigratory neural crest. In assays in *Xenopus* embryos, Cv-2 can inhibit BMP4 activity, consistent with previous work, suggesting that CR repeat proteins act as BMP antagonists. However, in other assays in *Xenopus* embryos, Cv-2 increases BMP4 activity. Furthermore, elevated Cv-2 expression leads to premature migration of trunk neural crest in the chick embryo, indicative of increased BMP activity. We discuss the implications of these findings for roles of Cv-2 in the modulation of BMP activity and neural crest cell migration.

Materials and methods

Sequencing and sequence analysis

Clones were sequenced at the Advanced Biotechnology Centre (Imperial College, London, UK). Sequences were analysed for putative open reading frames using the MacVector (Oxford Molecular). Nucleic acid and protein sequence similarity searches were performed using the gapped BLAST algorithms accessed at http://www.ncbi.nlm.nih.gov. Protein family searches (Pfam) were performed at http://pfam.wustl.edu and http://www.tigr.org/tdb/.

Whole-mount in situ hybridisation

Linearised cDNAs were used to synthesise digoxigenin labelled antisense RNA probes and in situ hybridisations performed using 'Protocol Four' as previously described (Xu and Wilkinson, 1998). In *Xenopus* blastomere injection experiments, Xbra transcripts were detected by in situ hybridisation and NBT/BCIP substrate, then alkaline phosphatase was acid-inactivated (Jowett, 1998) and fluorescein dextran lineage tracer detected using alkaline phosphatase-conjugated anti-fluorescein antibody and Fast Red substrate.

Generation of recombinant protein and immunoprecipitation analysis

The production of recombinant protein was carried out using the *Drosophila* Expression System (DES; Invitrogen). Cv-2-and chordin-coding regions were isolated by PCR and each inserted into the pMT/BIP/V5-HisA expression vector. Expression constructs were (co-)transfected into Schneider 2 insect cells (S2) using the calcium chloride technique (Invitrogen). Cell lysis, immunoprecipitation and western blotting were performed as described (Henkemeyer et al., 1994; Larrain et al., 2000a). Briefly, expressing cells were lysed with 2 ml PLC buffer, and cell debris removed by centrifugation. Protein G-Sepharose beads, pre-incubated with mouse anti-Myc antibody (Santa Cruz Biotechnology), were added to the samples and incubated overnight at 4°C. The beads were pelleted, washed, boiled in 20 μl loading buffer and then electrophoresed through 10% SDS polyacrylamide gels under reducing conditions. The proteins were electroblotted onto PDVF membranes and probed using the anti-V5 HRP conjugated antibody (1:5000).

Xenopus embryo manipulation

Synthesis of capped mRNA from cDNAs in pCS2 vector for in vivo injection was carried out as previously described (Moon and Christian, 1989). In some experiments, RNA was co-injected with lysinated fluorescein dextran lineage tracer. Embryos staged according to Nieuwkoop and Faber (Nieuwkoop and Faber, 1967) were used either for animal cap explant experiments or fixed for in situ hybridisation.

Animal cap explants were excised from stage 8-9 embryos that had been injected with the appropriate RNA(s) and allowed to develop to the desired stage. Total RNA was isolated using Trizol reagent (Gibco BRL) and was treated with RQ1 DNase (Promega) to remove genomic DNA contaminants according to supplier's specifications. cDNA was synthesised using MMLV RTase (Gibco BRL) and random primers (Promega). The PCR reaction mix contained 100 μM of each dNTP, 1×AmpliTaq buffer (Perkin Elmer), 1.5 mM $MgCl_2$, 0.1 μg of each primer, 1 μC ^{32}P dCTP and 1.25 units AmpliTaq DNA Polymerase (Perkin Elmer). PCR conditions of 25 cycles: 93°C for 30 seconds; 55°C for 1 minute; 72°C for 30 seconds. The PCR products were detected by electrophoresis on polyacrylamide gels followed by autoradiography or using a phosphoimager for quantitative analysis. Primer sequences used are as previously described (Domingos et al., 2001; Hemmati-Brivanlou et al., 1994; Ruiz i Altaba and Melton, 1989):

NCAM, CACAGTTCCACCAAATGC, GGAATCAAGCG-GTACAGA; BF-1, CCTCAACAAGTGCTTCGTCA, TAAAGGT-GAGTCCGGTGGAG; muscle actin, GCTGACAGAATGCAGAAG, TTGCTTGGAGGAGTGTGT; EF-1α, CAGATTGGTGCTGGATAT-GC and ACTGCCTTGATGACTCCTAG; Xbra, CACCGAGAAG-GAGCTGAAGGTTAG and TGCCACAAAGTCCAGCAGAACC.

Chick electroporations

Electroporation was carried out as previously described (Itasaki et al., 1999) using Cv-2-coding region cloned into pCS2 vector, and pCIG to express GFP. Briefly, after co-injection of pCS2Cv-2 and pCIG (3:1) into the neural tube lumen one 25 msecond square wave pulse of 25 mV was applied to the embryo using a pulse generator, the embryo allowed to recover for 1 minute before being sealed with parafilm, and re-incubated. The right side of the neural tube of stage 10–/11– embryos was electroporated and embryos dissected out 15 hours later at stage 16. The embryos were fixed in 4% PFA for 1 hour at room temperature and whole-mount in situ hybridisation carried out.

Results

Identification and structure of a novel chick CR domain gene

Using a chick embryo hindbrain cDNA library subtracted with pregastrulation stage cDNA (Christiansen et al., 2001), we identified in an in situ hybridisation screen a clone corresponding to an mRNA with a dynamic expression pattern in the dorsal neural tube. Sequencing of a full-length clone obtained from a neurula stage chick embryo cDNA library revealed that this clone encoded a polypeptide with an N-terminal signal peptide and five cysteine-rich (CR) motifs related to the Von Willebrand factor type C domain, and at the C-terminal end a Von Willebrand factor type D domain (VWD) (Fig. 1). CR motifs are found in a diverse group of proteins, some of which, including chordin, have been shown to bind BMPs via specific CR domains (Garcia Abreu et al., 2002; Larrain et al., 2000b). The VWD domain is present in a number of extracellular proteins, and may be involved in multimerisation via disulphide crosslinks through a conserved CGLCG motif (Fig. 1). The strongest sequence similarities are with two proteins that contain CR and VWD motifs (Fig. 1): *Drosophila* crossveinless 2 (cv-2), which has the same primary structure of five CR repeats and a C-terminal VWD motif (Conley et al., 2000); and kielin, which has 27 CR repeats and a C-terminal VWD motif (Matsui et al., 2000). Genome and cDNA database searching and sequence comparisons reveals a single *cv-2*-related gene in the human and mouse, and the structure and expression pattern of the mouse homologue has recently been described (Coffinier et al., 2002). We have therefore identified the chick homologue of Drosophila Cv-2, which we designate Cv-2. Biochemical and functional studies have suggested that a number of CR proteins, including chordin and procollagen IIA, bind to and antagonise BMP4 activity by sequestering BMP4 from its receptor (Larrain et al., 2000b). By contrast, *cv-2* was identified as a gene that promotes the activity of *dpp* (a *Drosophila* BMP homologue) required for the formation of crossveins in the *Drosophila* wing (Conley et al., 2000). We therefore set out to analyse potential relationships between Cv-2 and BMP signalling: first, by analysis of the expression pattern of Cv-2; and, second, by testing effects of Cv-2 on BMP activity in in vitro and in vivo assays.

Cv-2 expression

Cv-2 is expressed in a number of tissues during early chick embryogenesis. During gastrulation and neurulation, Cv-2 transcripts are detected in mesoderm ventrolateral to the primitive streak, as well as in dorsal neural tube of the hindbrain and spinal cord (Fig. 2A-D,G,H). At stage 13, expression is detected in the outflow tract of the heart, in the posterior part of the otic vesicle, adjacent to the dorsal aorta and in mesenchyme adjacent to the eye (Fig. 2E). By stage 19, Cv-2 expression also occurs in intersomitic mesenchyme, in nephrogenic mesenchyme and in sympathetic ganglia that are forming adjacent to the dorsal aorta (Fig. 2F,I,J).

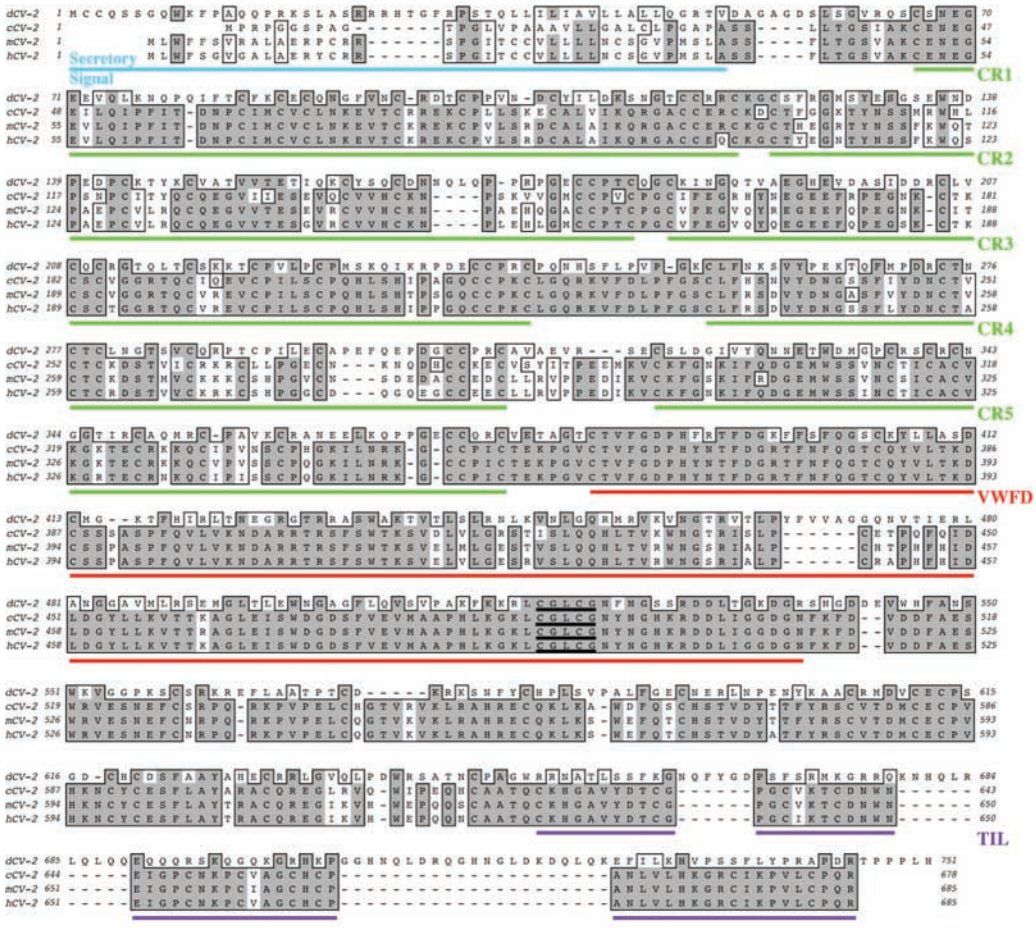

Fig. 1. Sequence of chick Cv-2. Amino acid sequence alignment of chicken, mouse, human and *Drosophila* Cv-2 with the ClustalW multiple sequence alignment using the BLOSUM30 algorithm. Identical and conserved amino acid substitutions are boxed, the former being identified by dark shading. Protein motifs identified by Pfam are coloured as follows: secretory signal in light blue; CR 1-5, cysteine-rich motif in green; VWFD, von Willebrand Factor type D domain in red; and TIL, trypsin inhibitor-like cysteine-rich domain in purple. The black bars highlight the amino acid sequence CGLCG, a motif for potential multimerisation by disulphide crosslinking. GenBank Accession Number AY731507.

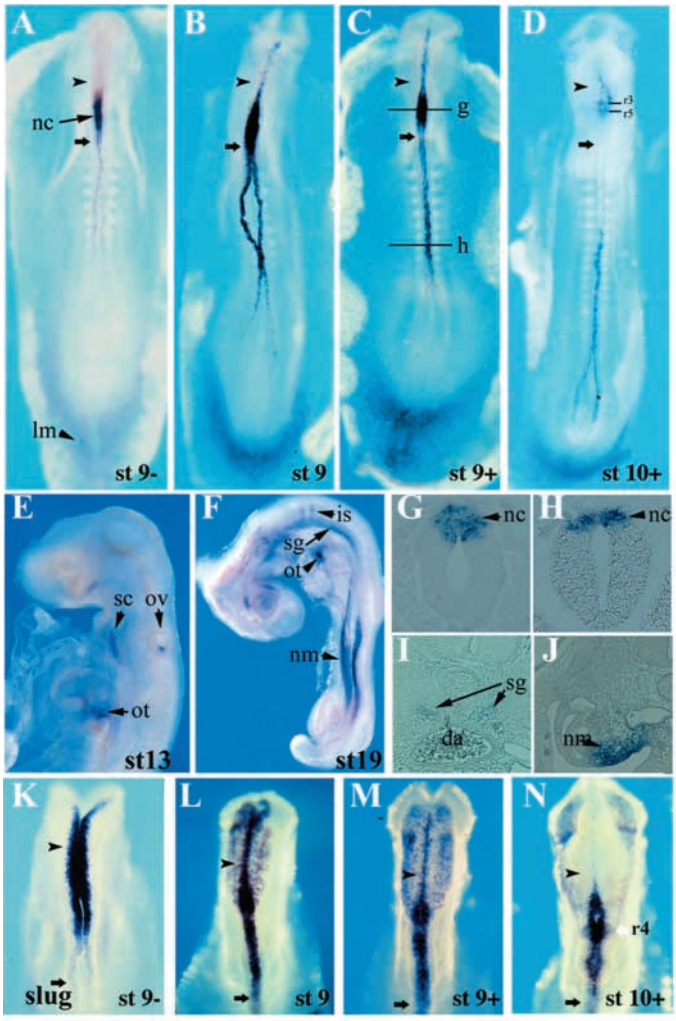

Fig. 2. Expression pattern of Cv-2 in the chick embryo. Whole-mount in situ hybridisations of Cv-2 (A-F) and Slug (K-N) and transverse sections of Cv-2 hybridised embryos (G-J). (A) HH stage 9– (seven-somite stage). Cv-2 expression is detected in posterior lateral mesoderm (lm) and in premigratory neural crest (nc) in the hindbrain and anterior spinal cord. (B) Stage 9 (eight somites). Cv-2 expression is upregulated in premigratory neural crest in the hindbrain and spinal cord, and in some cells in the midbrain. (C) Stage 9+ (10 somites). Expression seen at stage 9 persists but (D) by stage 10+ (13 somites) Cv-2 expression in the hindbrain and anterior spinal cord is downregulated. Low levels of Cv-2 expression occur in the hindbrain in rhombomeres 3 and 5. (E) Stage 13. Cv-2 transcripts are detected at the posterior lip of the otic vesicle (ov), the outflow tract of the heart (ot) and sclerotome surrounding the dorsal aorta between the branchial arches (sc). (F) Stage 19. Cv-2 transcripts are detected in the intersomitic region (is), in sympathetic ganglia (sg), in the outflow tract of the heart (ot) and in nephrogenic mesenchyme (nm). (G,H) Transverse sections through the neural tube of stage 9+ as indicted in C show that Cv-2 transcripts are restricted to premigratory neural crest cells (nc) in the dorsal neural tube. (I,J) Transverse sections through a stage 19 embryo, showing Cv-2 expression in (I) sympathetic ganglia (sg) adjacent to the dorsal aorta (da) and in (J) nephrogenic mesenchyme (nm) surrounding the nephric ducts. (K-N) In situ hybridisation to detect slug transcripts, a marker of premigratory and migrating neural crest from stage 9– to stage 10+. Black arrowheads identify the midbrain/hindbrain boundary, black arrows indicate the posterior limit of the hindbrain.

Many of these sites of *cv-2* gene expression correlate with regions of elevated BMP signalling (see Discussion), including the dorsal neural tube from which neural crest cells migrate, and the dorsal aorta adjacent to which they differentiate into sympathetic ganglia. As there is evidence that BMP4 signalling plays a role in these processes, we analysed Cv-2 expression in neural crest in more detail. In the dorsal hindbrain, Cv-2 RNA is upregulated at stage 9– (Fig. 2A) and high levels are maintained until stage 10 (Fig. 2B,C). By stage 10+ (Fig. 2D), Cv-2 expression has been downregulated to low levels in the hindbrain, and transcripts are localised to rhombomeres 3 and 5. Cv-2 expression occurs transiently and in a punctate pattern in the dorsal midbrain and forebrain between stages 9 to 10 (Fig. 2B,C). In addition, an anterior to posterior wave of Cv-2 expression occurs in the dorsal spinal cord starting at stage 9 (Fig. 2B), such that, by stage 10+, Cv-2 expression has been downregulated in the anterior trunk but is still expressed posteriorly (Fig. 2D). Comparison of Cv-2 with *slug*, a marker of premigratory neural crest cells (Fig. 2K-N), reveals that the upregulation of Cv-2 expression occurs at the same time or shortly after *slug* expression in the hindbrain and spinal cord, and prior to the onset of neural crest emigration. By contrast, Cv-2 expression occurs later than *slug* expression in the midbrain and forebrain.

Binding and modulation of BMP4 activity by Cv-2

To test whether Cv-2 protein can bind to BMP4, we used an expression system in *Drosophila* S2 cells to express V5 epitope-tagged Cv-2 protein or, as a positive control, V5-tagged chordin. S2 cell lysates were mixed with BMP4 protein, immunoprecipitated with anti-BMP4 antibody and a western blot probed with anti-V5 antibody. We found that Cv-2 protein co-immunoprecipitated with BMP4 protein (Fig. 3A).

We next investigated whether Cv-2 is an antagonist or promoter of BMP4 signalling, and used the well-characterised assays of RNA injections into *Xenopus laevis* embryos in which the level of BMP activity affects axis formation, neural induction and expression of mesodermal markers. Injection of 750 pg Cv-2 RNA into the dorsal blastomeres of four-cell stage embryos does not affect embryo development (45/45 embryos), but injection into ventral blastomeres leads to formation of a partial secondary axis (25/69 embryos; Fig. 3B-E) or a decreased body length (21/69 embryos; not shown). These effects of Cv-2 are similar to the dorsalising activity of noggin, but injection of 750 pg Cv-2 RNA was less potent than 25 pg of noggin RNA (31/34 embryos strongly dorsalised; data not shown), suggesting that Cv-2 acts as a weak antagonist of BMP activity.

To further investigate an antagonist activity, we microinjected Cv-2 RNA into the one-cell stage embryo followed by the detection of neural gene expression in isolated animal caps. Animal caps excised from stage 8 *Xenopus* embryos and cultured in isolation form non-neural ectoderm. Expression of BMP antagonists such as noggin in the animal caps leads to formation of neural ectoderm, as revealed by expression of the general neural marker NCAM and anterior neural marker BF-1. Induction of NCAM and BF-1 in animal caps does not occur following injection of increasing doses up to 4 ng of Cv-2 RNA, whereas injection of 0.1 ng of noggin RNA induces neural gene expression (Fig. 3F). However, co-injection of 2 ng Cv-2 RNA was found to increase the induction

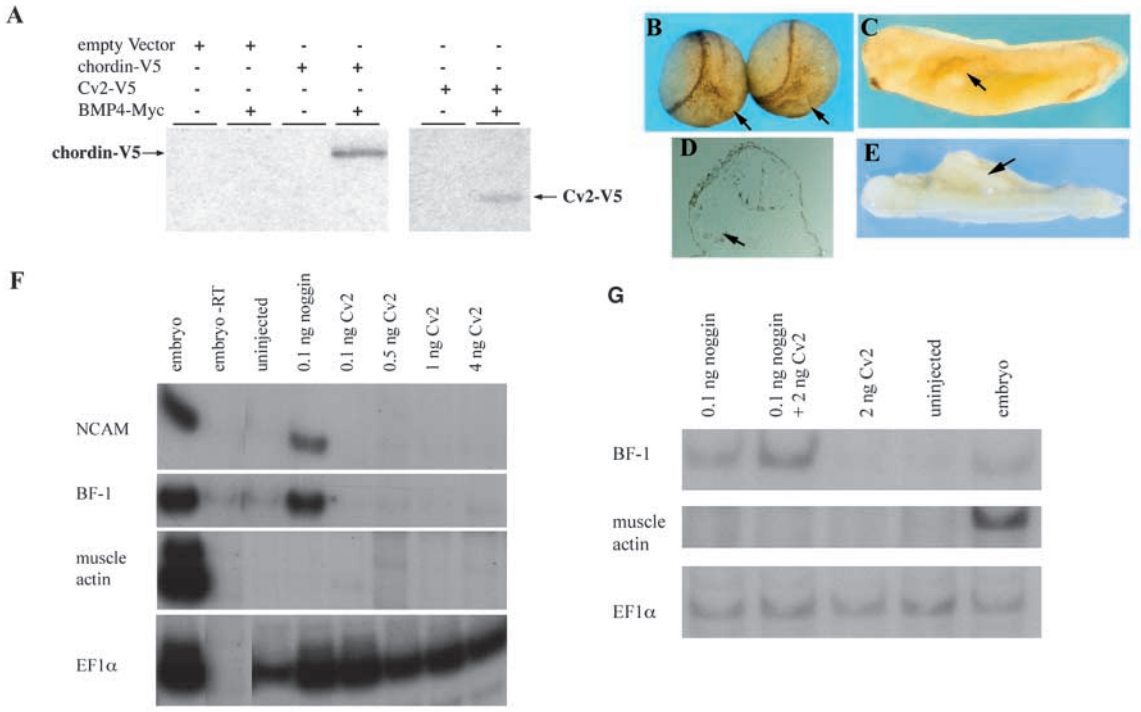

Fig. 3. In vitro analysis of Cv-2 activity. (A) Western blot analysis and co-immunoprecipitation reveals the direct interaction of V5-tagged Cv-2 protein with recombinant human BMP4 protein. V5-tagged chordin is used as a positive control for BMP binding. (B-E) Effect of Cv-2 injection on axis formation in *Xenopus* at stage 14 (B) and stage 27 (C-E). Ventral injections of Cv-2 RNA induce a partial secondary axis (arrows; B,C,E). Transverse section through the trunk of a stage 27 embryo injected ventrally with Cv-2 confirms the presence of an ectopic secondary neural epithelium (arrow, D). (F,G) RT-PCR analyses of *Xenopus* animal cap explants. (F) *Xenopus Noggin* mRNA induces expression of the general neural marker NCAM and anterior neural marker *BF1*. Increasing amounts of Cv-2 mRNA do not induce expression of either neural marker. (G) Co-injection of 2 ng Cv-2 RNA plus 0.1 ng noggin RNA increases the induction of BF-1 when compared with injection of 0.1 ng noggin RNA alone. The mesodermal marker muscle actin is used to confirm the absence of mesoderm in the explants and elongation factor-1 alpha (*EF-1α*) is used to assess the relative amounts of recovered mRNA.

of neural marker expression following injection of 0.1 ng noggin RNA (Fig. 4G). Thus, in these assays Cv-2 does not antagonise BMP4 activity sufficiently for neural induction to occur, but can synergise with a strong BMP antagonist.

In RNA-injection experiments, expression of Cv-2 protein is predicted to be at high levels in comparison with endogenous BMPs. Because the relative levels of Cv-2 and BMP proteins may influence BMP activity, we tested the effect of co-expressing Cv-2 and BMP4 on the regulation of a BMP-inducible gene. In one series of experiments, we used an assay (Cui et al., 2001) in which injection of BMP4 RNA into single animal pole blastomeres at the 32-cell stage induces the ectopic expression of the *Xbra* gene. At stage 10.5, *Xbra* expression is confined to the blastopore lip in uninjected embryos (Fig. 4A). However, in embryos ectopically expressing BMP4 or BMP4 plus Cv-2, ectopic expression of Xbra occurs, either in a domain co-extensive with normal Xbra expression around the blastopore lip (Fig. 4B,C) or as an isolated domain (Fig. 4D). Injection of increasing doses of BMP4 RNA, from 100 pg to 2 ng, led to ectopic Xbra expression in an increasing proportion of embryos (Fig. 4E). Co-injection of Cv-2 RNA increased the induction of Xbra expression at each dose of BMP4, whereas Cv-2 injection alone did not induce Xbra expression (Fig. 4E). For example, 200 pg BMP4 RNA plus 2 ng Cv-2 RNA is more potent than a tenfold higher amount of BMP4 RNA alone.

Based upon these results with expression in whole embryos, we then tested whether Cv-2 modulates the ability of BMP4 to induce Xbra expression in isolated animal caps (Ohkawara et al., 2002). Co-injection of 200 pg BMP4 RNA plus 200 pg Cv-2 was found to induce a fourfold higher level of Xbra expression compared with injection of 200 pg BMP4 RNA alone (Fig. 4F). Taken together, these results suggest that Cv-2 potentiates the effects of BMP signalling under these assay conditions.

Modulation of BMP activity in chick neural crest

The results of ectopic expression assays in *Xenopus* embryos raises the important issue of whether Cv-2 acts to inhibit or enhance BMP activity at endogenous sites of expression. One such site is in the dorsal neural tube from which neural crest cells migrate. In this location, threshold levels of BMP signalling are required for neural crest cell emigration, as inhibition of BMP4 activity delays migration, thus causing an anterior shift of the AP location at which migration is initiated (Sela-Donenfeld and Kalcheim, 1999). We therefore tested whether the onset of trunk neural crest migration could be altered by increasing the protein levels of Cv-2 via electroporation of an expression construct. Using Sox10 and HNK1 as neural crest markers, we found that increased expression of Cv-2 led to premature migration of neural crest, at a location two to three somites more posterior than on the

control non-electroporated side of the neural tube (9/16 embryos; Fig. 5C-E), whereas electroporation of empty vector had no effect (Fig. 5A,B). This finding suggests that increased expression of Cv-2 increases the effective level of BMP activity.

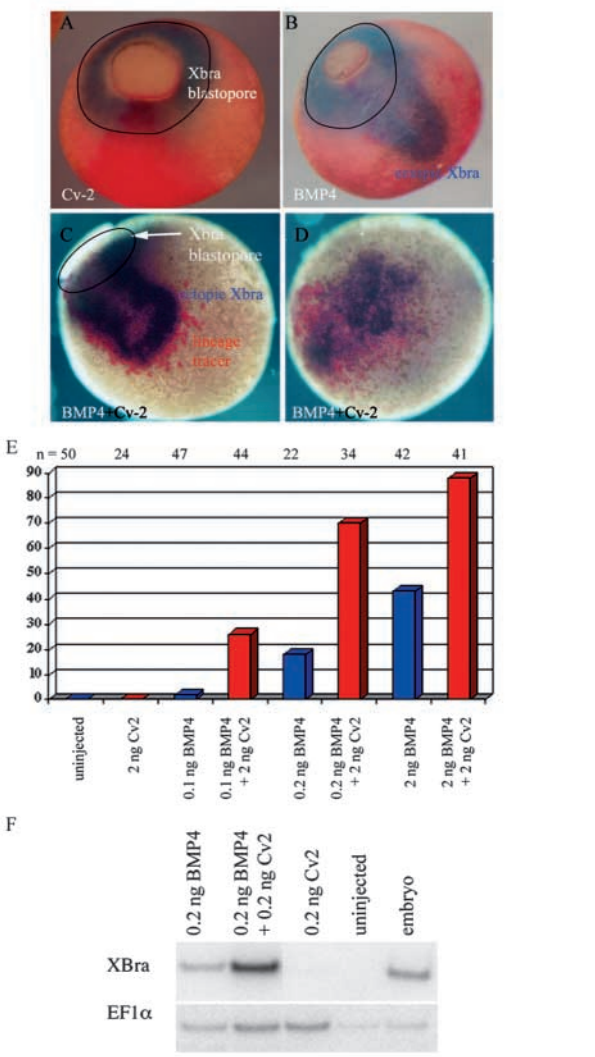

Fig. 4. Elevation of BMP4 activity by Cv-2. (A-D) Injection of Cv-2 (A), BMP4 (B) or BMP4 plus Cv-2 mRNA (C,D) with fluorescein dextran lineage tracer into a single animal pole blastomere at the 32-cell stage embryo, and Xbra expression detected at stage 10.5. Fluorescein dextran is detected in red, and Xbra in blue, with the normal expression domain around the blastopore lip indicated with a black ellipse. Ectopic BMP4 with or without Cv-2 induces ectopic expression of Xbra, either co-extensive with the normal expression around the blastopore lip (B,C) or as an isolated patch (D). (E) Summary of the effects of BMP4 and Cv-2 injections on the proportion of embryos with ectopic Xbra expression (%, *y*-axis). For each amount of BMP4 RNA, co-injection of Cv-2 RNA increases the induction of Xbra expression. The number of embryos analysed are indicated at the top of the chart. (F) Injection of BMP4 and/or Cv-2 mRNA at the one-cell stage embryo, followed by excision of animal caps at stage 8-9, and RT-PCR analysis of Xbra transcripts at stage 10.5. Quantitation with a phosphoimager reveals that Xbra expression is fourfold higher in BMP4 plus CV-2 injected animal caps than with BMP-4. Cv-2 alone does not induce Xbra expression.

The above experiments were performed at trunk levels. Cv-2 is also expressed in premigratory neural crest cells in the hindbrain region, where BMP4 has been proposed to promote apoptosis in branchial neural crest (Graham et al., 1994). However, overexpression of Cv-2 had no apparent effect on apoptosis in this region (data not shown).

Discussion

We have identified chick Cv-2, a homologue of *Drosophila* crossveinless-2, with a conserved structure of five CR repeats and a C-terminal Von Willebrand type D domain. As genetic studies show that crossveinless-2 promotes Dpp activity in the Drosophila wing (Conley et al., 2000), it might be anticipated that Cv-2 has an analogous role in vertebrate development in promoting BMP activity. Indeed, Cv-2 is found in a number of tissues that require high levels of BMP activity: in ventrolateral mesoderm (Streit and Stern, 1999), premigratory neural crest (Graham et al., 1994; Sela-Donenfeld and Kalcheim, 1999), the outflow tract of the heart (Delot et al., 2003), nephrogenic mesenchyme (Obara-Ishihara et al., 1999), and in sympathetic ganglia (Schneider et al., 1999). In many of these tissues, such as ventrolateral mesoderm and premigratory neural crest, Cv-2 expression occurs within a subdomain of BMP4 expression. In other locations, Cv-2 and BMP expression occurs in adjacent tissues; for example, BMPs expressed in the dorsal aorta induce differentiation of the sympathetic ganglia that express Cv-2. The pattern of Cv-2 in chick is similar to that described in a gene expression study of mouse Cv-2 (Coffinier et al., 2002), and is consistent with the possibility that vertebrate Cv-2 acts to elevate BMP activity. However, several CR-containing proteins can antagonise BMP activity by binding BMPs and sequestering them from BMP receptors (Garcia Abreu et al., 2002; Larrain et al., 2000b; Matsui et al., 2000). This therefore raises the issue of whether chick Cv-2 can increase and/or decrease BMP activity.

Antagonism and promotion of BMP4 activity

The *Xenopus* embryo is an important assay system for the study of BMP antagonists, in which inhibition of endogenous BMP activity leads to ectopic axis formation in whole embryos, dorsalisation of mesoderm or neural induction in animal caps. Such assays have revealed that a number of proteins containing CR repeats such as chordin, procollagen IIA and kielin can bind to and antagonise BMP4, suggesting a general role of CR domain proteins in decreasing BMP activity in vivo (Garcia Abreu et al., 2002; Larrain et al., 2000b). Chordin is a strong antagonist that when ectopically expressed dorsalises mesoderm and induces neural ectoderm (Piccolo et al., 1996), consistent with its role as a component of the BMP antagonist activity of the Spemann organiser. Procollagen IIA has also been proposed to act an antagonist of BMP activity, as ventral expression induces a secondary axis and dorsalises explanted mesoderm (Larrain et al., 2000b). Similarly, ectopic expression of kielin expands neural ectoderm in the embryo and dorsalises mesoderm (Matsui et al., 2000). However, kielin does not induce neural tissue in isolated animal caps, and it was therefore proposed that the expansion of neural ectoderm in the intact embryo could occur via the dorsalisation of mesoderm that in turn expresses neural inducing signals (Matsui et al., 2000). These findings are potentially relevant to

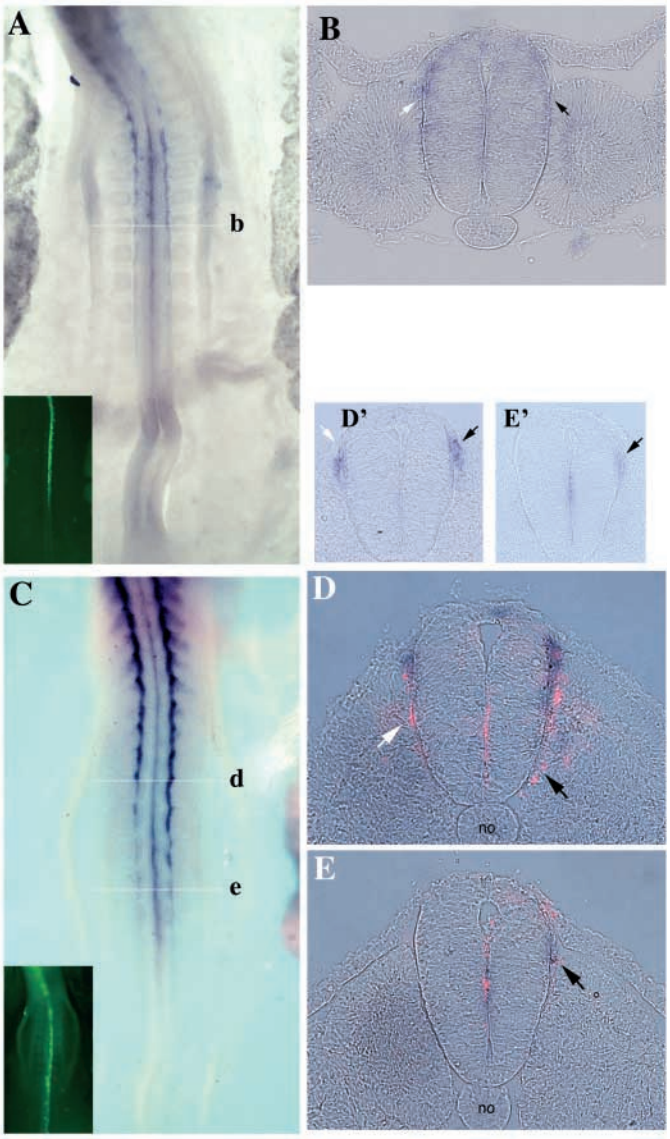

Fig. 5. Effect of elevated Cv-2 expression on trunk neural crest cell migration. (A-E) Representative images of the trunk region of stage 16 chick embryos electroporated with pCIG (A,B) or co-electroporated with pΔCS2Cv-2 and pCIG (C-E) expression constructs at HH stage 10+. (A) In situ hybridisation with Sox10 of a control pCIG electroporated embryo. (B) Transverse section of embryo (A) at axial level indicated; black and white arrows identify equal progression of Sox10-positive migratory neural crest cells on control and electroporated sides. (C) In situ hybridisation with Sox10 of an embryo overexpressing Cv-2. The domain of Sox10 expression extends further caudally on the side overexpressing Cv-2 (right) than the control side (left). Insets in A and C show the distribution of GFP-positive cells, demonstrating the domain and efficacy of the electroporation prior to in situ hybridisation analysis; left side of the neural tube is the control; right side is the electroporated side. (D,E) Transverse sections through embryo (in C) as indicated by lines, with immunofluorescence labelling of HNK1. (D′,E′) Corresponding bright-field images in which the Sox10 signal is more easily seen. In the more anterior section (D,D′) there is an increase in the number and distance of migration of HNK-1-positive migratory crest cells on the transfected side (black arrow) compared with the control side (white arrow). Sox10 signal is not detected in the ventral HNK1-expressing neural crest, perhaps owing to a decreased level of expression during ventral migration. In the more posterior section (E,E′) Sox10- and HNK-1-positive crest cells have initiated migration on the Cv-2 electroporated side (black arrow) but not on the untreated side.

Cv-2, as kielin protein has a related structure of CR repeats and a C-terminal VWD domain, and because, similar to kielin, we find that overexpression of Cv-2 induces ectopic neural tissue in the whole embryo, but not in animal caps. A difference is that kielin does not induce a secondary axis, whereas Cv-2 can do so. By analogy with the results of assays using individual CR domains (Larrain et al., 2000b), this may reflect that secondary axis formation requires a greater inhibition of BMP activity than does dorsalisation without secondary axis formation. A key question is why does Cv-2 inhibit BMP activity in these assays, whereas in other assays Cv-2 can increase BMP activity?

Our findings suggest that, when overexpressed, Cv-2 acts as a weak antagonist that cannot decrease BMP activity sufficiently to induce neural ectoderm in animal caps. The antagonistic effects of Cv-2 are detected in experiments in which overexpression of Cv-2 inhibits endogenous BMPs, whereas we find that Cv-2 can promote BMP activity when co-expressed with BMP4. Our findings are consistent with a model in which there is competition between binding of BMP4 to unoccupied Cv-2 (not already bound to BMP4) or to BMP receptor. When Cv-2 and BMP4 are present at similar levels, there is a low amount of unoccupied Cv-2, and thus BMP4 released from Cv-2 will frequently bind to BMP receptor. However, when Cv-2 is present in great excess over BMP4, the equilibrium shifts such that BMP4 will more frequently bind to unoccupied Cv-2 rather than to BMP4 receptor. One interpretation of our findings is that Cv-2 could have a dual role by decreasing the activity of low levels of BMP4, but increasing the activity of higher levels of BMP4. Alternatively, antagonism of BMP4 may be an effect of high levels of Cv-2 compared with BMP4 expression that do not occur in vivo.

There are similarities and differences between our results on chick Cv-2 and a recent study of a mouse Cv-2 homologue (Moser et al., 2003). Consistent with our results, expression of mouse Cv-2 was found to bind BMPs, to dorsalise mesoderm in activin-induced animal caps, and to induce secondary axis formation following ventral overexpression in *Xenopus* embryos. In addition, mouse Cv-2 antagonised the response to recombinant BMP4 in cell culture assays. However, these authors did not analyse neural induction in animal caps or carry out assays that detected cooperation with co-expressed BMP4, and consequently concluded that Cv-2 normally acts as an antagonist. Although the results are consistent with our findings that we interpret as a weak BMP antagonistic effect of overexpressed Cv-2, an apparent discrepancy is that expression of an excess of Cv-2 blocked the ability of BMP4 to ventralise the *Xenopus* embryo when expressed in dorsal tissue. By contrast, we find that Cv-2 increases the activity of co-expressed BMP4. However, we also find that overexpressed Cv-2 can cooperate with noggin, a strong BMP antagonist, to further block BMP activity. This may be due to the high ratio of unoccupied Cv-2 to BMPs in this situation, or alternatively it is possible that Cv-2 increases the binding of noggin to BMP4 (but see below). The inhibition by Cv-2 of BMP-

induced ventralisation of dorsal tissues may therefore be explained by cooperation between overexpressed Cv-2 and endogenous BMP antagonists that are present in dorsal regions.

Roles of Cv-2 in premigratory neural crest

Previous work has shown that inhibition of BMP activity by a posterior to anterior gradient of noggin controls the timing of neural crest cell emigration in the trunk (Sela-Donenfeld and Kalcheim, 1999). Neural crest migration is initiated when noggin expression in premigratory neural crest is downregulated by somite-derived signals (Sela-Donenfeld and Kalcheim, 2000), such that a threshold level of BMP activity is achieved. Expression of Cv-2 occurs in a broad domain in premigratory neural crest that shifts in an anterior-to-posterior wave and precedes the initiation of neural crest emigration. Thus, the onset of neural crest migration is preceded by an upregulation of Cv-2 and downregulation of noggin expression. Furthermore, elevated expression of Cv-2 leads to premature emigration of neural crest, consistent with Cv-2 acting to enhance BMP activity. This result argues that the increased antagonism of endogenous BMPs in the presence of noggin plus Cv-2 in *Xenopus* assays is an effect of overexpression, and that at physiological levels of expression, noggin and Cv-2 have opposite effects on BMP activity. We therefore propose that the timing of neural crest cell migration is regulated by the balance between the promotion of BMP activity by Cv-2 and inhibition by noggin. This model raises the question of how Cv-2 expression is controlled in trunk neural crest, and whether it is downstream of the slug transcription factor that regulates neural crest cell migration, or whether, like noggin, Cv-2 expression is controlled by signals from the adjacent somites.

BMP4 appears to have a distinct role in branchial compared with trunk neural crest in the chick embryo, in which it regulates the elevated apoptosis of neural crest cells derived from rhombomeres r3 and r5 (Graham et al., 1994), and is antagonised by noggin expression in r4 (Smith and Graham, 2001). The expression of Cv-2 in premigratory branchial neural crest could underlie a promotion of BMP4 activity in opposition to noggin, and intriguingly at late stages of expression in the hindbrain, Cv-2 transcripts are restricted to dorsal r3 and r5. Although we did not detect altered apoptosis following ectopic expression of Cv-2, it is possible that this reflects a lack of sensitivity of this response to any changes in BMP4 activity, in contrast to the threshold levels required for trunk neural crest migration. Alternatively, BMP4 may have an indirect role in the apoptosis of branchial neural crest (Farlie et al., 1999).

Potential mechanisms of Cv-2 action

Our findings provide the first evidence that vertebrate Cv-2 elevates BMP4 activity, similar to *Drosophila* cv-2, whereas other studies of vertebrate Cv-2 and of related CR proteins had only detected an antagonistic activity. Loss-of-function studies will be required to determine whether Cv-2 has a dual role in promoting and blocking BMP activity or normally acts only to increase BMP activity. A key question is how Cv-2 promotes BMP activity. As suggested for Drosophila cv-2 (Conley et al., 2000), the presence of a VWD domain implicated in multimerisation of extracellular matrix proteins raises the possibility that Cv-2 binds to matrix and locally elevates BMP4 levels by constraining free diffusion into adjacent tissue; this requires that Cv-2 can release BMP4 to BMP receptor and acts as a ‘sponge’ rather than a ‘trap’ for BMP4. This model predicts that Cv-2 activity can only be detected in the context of spatially restricted BMPs, and can explain why Cv-2 does not elevate BMP activity in cell culture experiments (Moser et al., 2003). Furthermore, as BMP4 itself can bind to specific extracellular matrix proteins (Ohkawara et al., 2002), the ability of Cv-2 to modulate BMP activity may depend upon which matrix proteins are present within the tissue.

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